AD_____
(Leave blank)

Award Number:
DAMD17-03-1-0479

TITLE:
The impact of exercise on the vulnerability of dopamine neurons to cell death in animal models of Parkinson's disease

PRINCIPAL INVESTIGATOR:
Michael J. Zigmond, Ph.D.
Amanda Smith, Ph.D.

CONTRACTING ORGANIZATION:
University of Pittsburgh
Pittsburgh, PA 15260

REPORT DATE:
July 2005

TYPE OF REPORT:
Annual Report

PREPARED FOR: U. S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
15-07-2005	Annual	16-06-2004 – 15-06-2005

4. TITLE AND SUBTITLE
The impact of exercise on the vulnerability of dopamine Neurons to cell death in animal models of Parkinson's disease

5a. CONTRACT NUMBER
DAMD17-03-1-0479

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michael J. Zigmond, Ph.D.

Amanda Smith, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, PA 15260

E-mail: zigmondm@upmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
X Approved for public release; distribution unlimited

Distribution limited to U.S. Government agencies only; report contains proprietary information

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Parkinson's disease (PD) results in part from the loss of dopamine (DA) neurons. We hypothesize that exercise reduces the vulnerability of DA neurons to neurotoxin exposure, whereas stress increases vulnerability. We have outlined experiments to test this hypothesis in rats treated with one of several neurotoxins, beginning with 6-hydroxydopamine (6-OHDA). Over the past year, we increased the size and training of our research team and made a number of observations of direct relevance to our hypothesis. We also have requested permission to expand our objectives to include critical studies on the mechanism of the actions of exercise, using both in vivo and in vitro approaches. Our focus will be on the actions of trophic factors and the roles played by intracellular signaling cascades.

15. SUBJECT TERMS
Parkinson's disease, dopamine, exercise, neuroprotection, basal ganglia

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Michael J. Zigmond
Unclassified	Unclassified	Unclassified	Unlimited	9	19b. TELEPHONE NUMBER *(include area code)* 412-624-4258

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction:

Parkinson's disease (PD) results in part from the progressive loss of dopamine (DA) neurons projecting from substantia nigra (SN) to striatum. Although the cause of this neurodegenerative process is unknown, candidates include oxidative stress, mitochondrial dysfunction, and excitotoxicity. These are likely to result from exposure to environmental toxins, perhaps coupled with one or more bases for increased vulnerability. Such increased vulnerability could include genetic predisposition, emotional or physical stress, or exposure to certain recreational drugs. We have developed the hypothesis that exercise *reduces* the vulnerability of DA neurons to neurotoxin exposure under basal conditions and blocks stress-induced exacerbation of toxin-induced DA neuron loss.

In this series of studies, adult male rats are to be given one of several exercise regimens prior to toxin exposure. Behavioral, biochemical, and histological analyses will be used to determine (1) whether the neuroprotective effects of cast-induced limb use in rats treated with 6-hydroxydopamine (6-OHDA) are also seen with other forms of exercise; (2) how much exercise is required, when must it occur, and how permanent are the effects; (3) if exercise also protects against the increased vulnerability to toxins caused by other stressors; and (4) the generality of our results with 6-OHDA to other toxins. We have recently requested an expansion of our Statement of Work (SOW) so that we can also include studies that explore the mechanism by which exercise and stress interacts to influence the vulnerability of DA neurons. Although we have not yet received official approval of that request, this progress report will briefly mention some of our progress in these areas.

Body:

1. Recruitment and training of staff

In 2003-4 we were able to recruit four individuals into our research group, each of whom has spent some of their time on this project. Those individuals are Michelle Fischer, the research assistant who has taken the lead in data collection, and three postdoctoral fellows, Amina El Ayadi, Rehana Leak, and Niklas Lindgren. Each of these individuals was trained to do small animal stereotaxic surgery, carry out neurological tests on rodents and to analyze the loss of dopamine (DA) neurons using both immunocytochemistry (ICC) and high performance liquid chromatography (HPLC). More recently, the group has expanded its skills to include the ability to measure trophic factor levels by ELISA and to measure MAP kinase levels via both ICC and Western blot analysis. Now a year later we have a highly functioning team, to which we have added Sandra Castro, a senior research assistant, and a group of individuals interested in in vitro models of the neuroprotective effects of trophic factors and MAP kinases.

2. Effect of physical therapy on the neuroanatomical response to 6-OHDA:

Animals exposed to unilateral infusion of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB) – the path taken by DA axons from the ventral mesencephalon to their targets in the corpus striatum – show an extreme motor asymmetry in their use of the affected limb and a unilateral loss of striatal DA. Just prior to the beginning of the current 4-year period of funding, we had shown that these effects could be blocked by placing a cast on the forelimb ipsilateral to the infusion of 6-OHDA for 7 days immediately before or after surgery (Tillerson et al., 2001; 2002; Cohen et al., 2003).

As a joint project between this grant and a grant from the National Institute of Neurological Disorders and Stroke (NINDS), we began immediately to determine if exercise *prior to* 6-OHDA administration reduced the loss of DA neurons in the nigra-striatal projection and, if so, whether this resulted from sprouting of residual neurons or a more fundamental reduction in the vulnerability of these neurons to the toxin. In our initial experiments, which were described in our last progress report, animals were forced to rely on one limb for 7 days and then 6-OHDA was infused into the ipsilateral MFB. Our results clearly indicated that the casting procedure increased the animal's use of its contralateral forelimb and dramatically reduced the vulnerability of the DA neurons in the ipsilateral SN to 6-OHDA. This conclusion was based on measures of behavior, tyrosine hydroxylase immunoreactive neurons, Nissl stain in the SN, and the uptake of Fluoro-jade B staining, a marker of degeneration. We also were able to extend our findings to a second group of DA neurons, those projecting from the ventral tegmental area to the nucleus accumbens (Cohen et al., 2004).

3. Use of micro-PET in our 6-OHDA model of PD

One of the problems that we and others have in studying interventions in animal models of PD is the inability to examine progressive biological changes in the same animal. We therefore have performed a pilot study to investigate the utility of microPET to monitor changes in DA terminals in rats given unilateral injections of 6-OHDA. The cost of the scans was covered by the PET center itself.

Rats received 6-OHDA (0.75-3.0µg) delivered into the MFB. After 7 or 28 days, the animals underwent PET imaging using ligands for the DA transporter (DAT) ([^{11}C] β-CFT) and/or the D2-receptor ([^{11}C] raclopride). DA release was assessed using D2 receptor imaging before and after amphetamine challenge (4 mg/kg i.p). The extent of striatal denervation was assessed by DA tissue content measured by HPLC. Data were expressed relative to the nonlesioned striatum. [^{11}C] β-CFT binding was reduced in lesioned striatum (range: -18 to -62%), with the reduction being correlated with dose of 6-OHDA and DA depletion. Modest increases in [^{11}C]raclopride binding in lesioned striatum relative to the nonlesioned side were observed in rats that received the highest dose of 6-OHDA (+16 and +35%), suggesting that D2 upregulation occurred only after pronounced DA denervation. Amphetamine pretreatment resulted in greater asymmetry in [^{11}C]raclopride binding between the lesioned and control striatum (range: +7% to +48%), suggesting attenuated DA release in the lesioned striatum. MicroPET was also able to detect a significant attenuation of DAT loss in animals subjected to forced limb use immediately following 6-OHDA infusion compared to lesioned animals that did not undergo motor therapy ($p<0.001$) (Lopresti et al., 2004). We conclude that microPET is useful for making non-invasive assessments of DA function in rodent models of PD, and may be applied in longitudinal studies to assess the response to putative therapies for PD. Unfortunately, our attempts to obtain funding to continue these studies have not been successful and they are currently on hold.

4. Ongoing studies to extend studies of casting in our model

Although the hypothesis that exercise reduces the behavioral and biological effects of DA-specific neurotoxins has now been supported by three papers from our group, our additional unpublished experiments, and a number of reports from several labs, we recently have not been able to replicate the effect. There are several possible reasons for this and we are currently examining each one. First, our animals may be experiencing more stress and, if so, this might be potentiating the effects of the toxin. Possible origins of the stress include an infection (our animal quarters has had a problem with pin-worms for several months) and the chemicals being

used to eradicate that infection, being housed in a room with female rats (due to a temporary shortage of space), and a tighter cast (employed to reduce the number of animals who are able to remove their cast). Second, we have noticed that our lesions have become somewhat larger – perhaps we have reached a lesion size against which exercise is ineffective. We hope to have resolved this issue within the next two months.

5. Additional studies of exercise

We have begun to examine the effect of treadmill running on the vulnerability of DA neurons in the SN against 6-OHDA. In our last progress report we indicated that we could not draw any definitive conclusions regarding this approach. There may have been two problems. First, the effect if any was small. This in turn could have been caused by our inability to get male Sprague-Dawley rats to run on a treadmill without the use of a mild shock to encourage them to move out of the start box during the training period. This may have resulted in enough stress to block the effects of the exercise. (Note comment on stress in the previous section.)

After consulting with two other researchers who have used treadmill running, we have decided to use female Long Evans rats, which are reported to require less coaxing to run on the treadmill than male rats previously used (Sprague Dawley). These studies have just been initiated and are quite promising. The animals appear to learn to run on the treadmill with little if any coaxing and the mild shock during training was unnecessary. We are currently determining whether treadmill exercise attenuates the effects of 6-OHDA in these animals.

6. Preconditioning with toxin exposure

As noted in our previous progress report, it has been shown in animal models of stroke and cardiac disease that exposure to small stressors for short durations can confer resistance to subsequent stressors in a variety of organs. Although not proposed in our original application, we felt that it would be instructive to determine if exposure to low levels of toxin could also cause “preconditioning” in a model of Parkinson's disease and proceeded to show that a very low dose of 6-OHDA (0.5 – 1.0 µg) could reduce the effects of a much larger dose of the toxins. Over the past year, we have pursued this preconditioning effect in an in vitro model. Using the DA cell line MN9D, we find that a brief exposure to a sub-toxic concentration of 6-OHDA (5-20 μM) resulted in a very large attenuation of the neurotoxin effects of a much large concentration of the toxin (Leak et al., 2004). This may be a general phenomenon, as we have also shown in the same cell line that 6-OHDA protected against the toxic effects of the proteasome inhibitor, MG132, and that methamphetamine protected against 6-OHDA. We are now exploring the mechanism of this effect.

7. Effects of 6-OHDA, exercise, and GDNF on protein kinase activity.

Given that exposure to 6-OHDA can protect against subsequent toxin exposure, we should be able to determine whether it triggered the activation of pro-survival signaling cascades. Our studies have involved both in vitro and in vivo models and have used both Western blot analysis and immunocytochemistry (ICC).

Our in vitro studies have employed the DA cell line MN9D. They indicate that 6-OHDA produced a rapid increase in the activity of the mitogen-activated protein kinase (MAPK), ERK1/2, which peaked at 15 minutes and had returned to baseline by 30-60 min. Blockade of the initial increase in pERK1/2 in MN9D cells potentiated 6-OHDA toxicity, suggesting that it

represented an attempt by the cells to protect themselves against the oxidative stress (Lin et al., 2004; 2005).

Next, 6-OHDA was injected along the MFB and increased activation of pERK1/2 was assessed in the SN and striatum after sacrificing our animals by focused microwave irradiation using facilities at the CDC in Morgantown, WV. Again, a combination of Western blot and ICC was used. The activation of ERK1/2 in response to 6-OHDA was observed in cells as early as 15 min in the SN and persisted for at least 24 hrs post-infusion. Double labeling for pERK1/2 and tyrosine hydroxylase (TH) revealed that the increase in pERK1/2 was in DA neurons in the SN. In the striatum, increases in pERK1/2 was first observed at 1 hr and persisted for at least 24 hrs. Activation of ERK1/2 was observed in fibers as well as cells in the striatum. We believe that the increase in pERK1/2 in fibers in the *striatum* is in TH immunoreactive terminals; however, double labeling is still needed to show co-localization of pERK1/2 and TH in the striatum. As in the case of the MN9D cells, we hypothesize that the activation of pERK1/2 within DA cells of SN and DA fibers within the striatum is a self-protective response to counteract the effects of 6-OHDA-induced oxidative stress. Experiments are ongoing to determine the precise role of pERK1/2 in oxidative-induced damage to the nigrostriatal pathway after infusion of 6-OHDA into the MFB (Smith et al., 2004; Castro et al., 2005).

We also observed an increase in pERK1/2 in cells within the dorsal striatum. Adult, male Sprague-Dawley rats received 6-OHDA (6 µg in 1 µl) into the striatum. This increase could be detected by 15 min and persisted for several hours. A similar temporal profile was observed for pJNK, although the peak increase was lower than for pERK. The morphology of the cells containing the increased activated MAP kinases suggests that they were the principal target neurons for the DA projection from SN, the medium spiny neurons, although this has not yet been confirmed by double labeling. The increases in MAP kinases were accompanied by a delayed increase in cfos, which was first detectable at 1 hr and remained for at least 3 hr. No changes in activated MAP kinases or cfos were observed in the substantia nigra (Smith et al., 2004, Fischer et al., 2005).

Activation of ERK1/2 and cfos induction has previously been reported following administration of drugs that activate DA receptors, and the induction of cfos can be attenuated by the administration of a DA antagonist. Thus, we hypothesize that changes in activated MAP kinases and in cfos represent the effects of endogenous DA being released in response to the neurotoxic actions of 6-OHDA. This suggests that initial changes in striatal pERK and cfos would be useful as short-term indices of the efficacy of neuroprotective treatments. Experiments are currently underway to test this hypothesis.

Key Research Accomplishments:

- Provided further training to staff; increased staff size
- Extended observations on forced limb use to analyses by micro-PET and to a non-motor DA projection.
- Began to expand the range of exercises used by employing a treadmill.
- Examined the defects of exercise on protein kinases.
- Demonstrated preconditioning by 6-OHDA and methamphetamine in an in vitro model.

- Examined the effects of 6-OHDA on protein kinases in DA neurons and their targets.

- Incorporated in vitro studies to further explore underlying mechanisms of the effects of trophic factors and the role of signaling cascades.

Reportable Outcomes:

- Both exercise and 6-OHDA alter the activity of the key protein kinases, ERK, Akt, and JNK. The increases in pERK appear to represent a protective response, at least in the case of 6-OHDA.

- Exposure to very low levels of 6-OHDA results in significant protection against higher levels of 6-OHDA exposure in an in vitro model.

Conclusions and Plans for Year 03:

We remain confident of our initial findings regarding the effects of forced exercise on the vulnerability of DA neurons to 6-OHDA. We feel that the problems we have been having with this paradigm will be resolved in the next two months, which will allow us to continue with our original plans. In anticipation of this we are in the process of expanding the types of exercise we will use to include treadmill and running wheels. We will also begin a study of the effects of other toxins and of the effects of stressors (particularly LPS) on DA neurons vulnerability.

We are aware that the model for PD that we use – acute 6-OHDA – may have many shortcomings, including the rapid rate at which it kills DA neurons (PD is gradual), the mechanisms of that death (which may differ from what occurs in PD), and the nature of its neuropathological effects (which are less broad than occurs in PD). At present we are exploring other models. In particular we are using the proteasome inhibitor PSI.

Thus far, we have both in vivo and in vitro data suggesting that exercise is neuroprotective by virtue of its ability to increase GDNF, which in turn increases the activity of the MAP kinase ERK, which reduces oxidative stress. However, this model is based primarily on correlations, and we now wish to begin a more rigorous test. This will involve the use of pharmacological inhibitors, neutralizing antibodies, transgenic and knockout mice, and molecular biological approaches.

Finally, we feel that the phenomenon of preconditioning may provide us with important insights into the capacity of the brain to protect itself against insult. Thus, we plan to examine the mechanism that underlies these effects.

References

Castro SL, Leak RK, Smith AD. Activation of MAPK in DA neurons after 6-hydroxydopamine into the medial forebrain bundle. Society for Neuroscience Meeting, Washington, DC, November 2005.

Cohen AD, Tillerson JL, Smith AD, Schallert T, Zigmond MJ. (2003) Neuroprotective effects of prior limb use in 6-hydroxydopamine-treated rats: possible role of GDNF. J Neurochem. 85:299-305.

Cohen, AD, El Ayadi A, Smith AD, Zigmond MJ. Forced limb use prior to 6-OHDA reduces vulnerability of dopamine neurons to oxidative stress. Program No. 221.1. 2004 Abstract Viewer and Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.

Fischer ML, Leak RK, Zigmond MJ, Smith AD. 6-Hydroxydopamine causes an acute increase in c-fos and MAP kinases in the rat striatum. Society for Neuroscience Meeting, Washington, DC, November 2005.

Leak RK, Lin E, DeFranco D, Smith AD, Zigmond MJ. Sublethal concentrations of 6-OHDA preconditions dopaminergic cells against lethal exposure. Program No. 562.21. 2004 Abstract Viewer and Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.

Lin E, Perez RG, Zigmond MJ. Role of ERK1/2 activation in dopaminergic cell survival in response to oxidative stress. Program No. 93.9. 2004 Abstract Viewer and Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.

Lin E, Perez RG, Zigmond M J. 2005. Effect of 6-OHDA on ERK activation in a dopaminergic cell line: Relation to cell survival. Submitted to J Biol Chem.

Lopresti BJ, Mathis CA, Mason NS C, Bohnen NI, Fischer M, Ruszkiewicz JA, Smith AD, Zigmond MJ. The use of positron emission tomography to measure dopamine nerve terminal degeneration. Program No. 562.14. 2004 Abstract Viewer and Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.

Smith AD, Slagel SL, Castro SL. Activation of MAP kinases by forced limb use in a 6-hydroxdopamine parkinsonian rodent model. Program No. 221.5. 2004 Abstract Viewer and Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.

Tillerson JL, Cohen AD, Philhower J, Miller GW, Zigmond MJ, Schallert T. (2001) Forced limb-use effects on the behavioral and neurochemical effects of 6-hydroxydopamine. J Neurosci. 21:4427-4435.

Tillerson JL, Cohen AD, Caudle WM, Zigmond MJ, Schallert T, Miller GW. (2002) Forced nonuse in unilateral parkinsonian rats exacerbates injury. J Neurosci. 22:6790-6799.